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(54) Title: BACTERIAL XYLANASE

(57) Abstract

The invention provides a bacterium, isolatable from wood compost, having the following characteristics: A. ability to grow at a temperature between 20 and 45°; B. ability to grow in the pH range of 5 to 9.5; C. ability to grow on Luria-Bertani agar at 37°; D. ability to grow under solid state or submerged culture conditions; and E. constitutive production and/or extracellular release of at least one xylanase having an associated cellulase activity of less than 0.1 %. A second aspect of the invention provides xylanase(s) produced by the bacterium. The xylanase is active at neutral to alkaline pH, has high thermal stability, and is useful in a variety of industrial applications. In a preferred embodiment, the xylanase of the invention is used in paper manufacture as a bleaching aid or bleach booster in the bleaching of kraft pulp.

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BACTERIAL XYLANASE

This invention relates to xylanase enzymes derived from bacteria, and in particular to xylanases which are free of any significant cellulase activity and which are active at high temperature and at neutral to alkaline pH. Xylanases having these characteristics are particularly useful in the bleaching of wood pulps, such as kraft pulps.

BACKGROUND OF THE INVENTION

Enzymes are proteins present in all living cells, where apart from controlling metabolic processes, they break down food materials into simpler compounds. The enzymes are catalysts which speed up processes which would otherwise proceed very slowly, or not at all. Moreover, enzymes are very specific, breaking down only one type of compound.

Xylan is a polysaccharide found in most plant cell walls, consisting of D-xylose units linked by β-1-4 glycosidic bonds. It occurs with another polysaccharide, cellulose and an amorphous binding polymer, lignin. Xylan forms a major component of plant hemicelluloses, and varies in the nature of substituents on the sugar groups, depending on the origin. For example, xylans derived from hardwoods typically consist of a backbone of O-acetyl-4-O-methylglucuronoxylan, in which about 10% of the xylose units carry 4-O-methylglucuronic acid side chains linked via α-1,2 bonds, and 70% of the xylose residues are acetylated at C-2 or C-3. In contrast, xylans derived from softwoods are usually arabino-4-O-methylglucuronoxylans in which over 10% of the xylose sub-units carry arabinofuranose residues linked via α-1,3 bonds. Enzymes which are able to degrade xylan are called xylanases (endo-1,4-β-D-xylanases; International enzyme nomenclature EC 3.2.1.8).

Commercial preparations of xylanase, often in combination with other cell wall degrading enzymes, have been used in the extraction or liquefaction of plant material. For example, in the food industry, the mashing process for the production of juices can be made to produce higher yields and better processing with the application of cell wall degrading enzymes, which include xylanase.

The primary source of cellulose for paper manufacture is wood, and may be either hardwood or softwood. The initial step in paper manufacture is the reduction of wood to the fibre state, which may be achieved by mechanical or chemical pulping methods. Chemical pulping involves the "cooking" of woodchips with chemical reagents in order to separate the cellulose fibres from the other wood components, and to break down the lignin and other extraneous compounds so that the cellulose is left in tact in its fibrous form. The most common process is the kraft or sulphate process, which can be applied to almost any timber species. The active ingredients are sodium hydroxide and sodium sulphide in a strongly alkaline solution.

During the kraft pulping process, xylan in the wood is initially dissolved in the pulping liquor, but with time, reprecipitates on to the resulting pulp. Wood lignin is modified and dissolved by the pulping liquors. However, about 10% of the lignin remains in the kraft pulp. To brighten the pulp, the lignin must be removed by bleaching chemicals, such as chlorine, which generate environmentally hazardous wastes.

More recently, commercial xylanase preparations have been used as an aid to the bleaching of kraft wood pulps. A program of cooperation between research institutes and the pulping industry has shown that treating the unbleached kraft pulp with xylanase results in a reduction in the amount of bleaching chemicals required to obtain a full brightness pulp. It is believed that xylanase acts as a bleaching aid (bleach booster) by

releasing some trapped residual lignin within the pulp matrix and giving better access to bleaching chemicals. It is widely believed that xylanase breaks down reprecipitated xylan which forms a coating on the pulp, thus releasing trapped residual lignin from within the pulp matrix, and allowing better access of bleaching chemicals to this matrix. Thus xylanase acts as a bleaching aid or bleach booster.

In the kraft process, the pulp is typically handled at high temperatures and neutral to alkaline pH. Commercial xylanases typically have a temperature optimum of about 50°C and a pH optimum of about 5, and are thus subject to rapid denaturation under process conditions. Thus there is a need for xylanases which are able to act optimally on the kraft pulp without any requirement to adjust the temperature or pH. In order to be useful as a bleaching aid, the xylanase must also be free of any significant cellulase activity, since cellulase would cause an undesirable loss of cellulose fibre.

In addition to its use in chemical kraft processes, xylanases are useful in the preparation of animal feedstuffs from raw materials having a high lignin content, and in the preparation of dough for bread making; see for example International Patent Publication No. WO 92/01793, and ligininolytic enzymes are useful in processes for enzymatic removal of printing ink from waste or scrap paper; see International Patent Publication No. WO 1/14820. A variety of uses for xylanases are discussed in Australian Patent Application No. 43479/93. The entire disclosures of these specifications are herein incorporated by reference.

We have screened microorganisms newly isolated from a range of environments in order to identify those which produce high levels of xylanases with high temperature optima and which are active at neutral to alkaline pH. A previously unidentified bacterium which we have designated B698, isolated from a wood compost,

produces such a xylanase in high yield and free of significant cellulase activity.

SUMMARY OF THE INVENTION

According to one aspect, the invention provides a bacterium, isolatable from wood compost, having the following characteristics:

A. Ability to grow at a temperature between 20 and 45°;

B. Ability to grow in the pH range of 5 to 9.5;

C. Ability to grow on Luria-Bertani agar at 37°;

D. Ability to grow under solid state or submerged culture conditions; and

E. Constitutive production and/or extracellular release of at least one xylanase having an associated cellulase activity of less than 0.1%.

Preferably xylanase production is enhanced by growth in the presence of xylan or of a lignocellulose substrate.

More preferably the at least one xylanase has at least one characteristic selected from the group consisting of activity at pH between 4.5 and 9, a thermal activity range up to 80°C, and high thermal stability up to 70°C. Most preferably the xylanase(s) produced by the bacterium is/are effective on both soluble and insoluble xylans.

In a particularly preferred embodiment, the bacterium has the characteristics of the bacterial isolate designated B698, as deposited under the provisions of the Budapest Treaty in the Australian Government Analytical Laboratories, PO Box 385, Pymble, New South Wales 2073, Australia, on 1 March 1994, under Accession No. N94/7647, or a mutant or derivative thereof having the ability to produce a xylanase as described above.

According to a second aspect, the invention provides a xylanase having the characteristics described above, and compositions comprising said xylanase as active ingredient, such as bleaching aids.

The invention also includes within its scope a process of bleaching kraft pulp comprising the step of using a xylanase according to the invention as a bleaching aid or bleach booster, and processes for removal of printing ink from paper, processes for preparing animal feed compositions, and methods of preparing dough for bread making, which processes comprise the step of using a xylanase according to the invention.

We have found that the bacterium B698, when grown under suitable fermentation conditions, will produce at least one xylanase which accumulates in the extracellular fermentation broth. The xylanase from such a broth has a thermal activity range from ambient up to 65°C and a useful pH range from 5 to 8, with optimal activity at pH 6 - 6.5. The xylanase has very high thermal stability, retaining 100% activity after 3 hrs and 90% activity after 22 hrs at 60°C. Cellulase activity associated with the xylanase is minimal (<0.1%).

While the following description refers to a single xylanase, our results indicate that there are in fact at least two different xylanases produced during fermentation of bacterium B698, and all xylanases produced by this organism are within the scope of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described by way of reference only to the following non-limiting examples, and to the figures, in which:

Figure 1 shows the variation of activity of xylanase from bacterium B698 with pH, and

Figure 2 illustrates the variation in activity of xylanase from bacterium B698 with temperature.

Example 1

A bacterium which we have designated B698 was isolated from a sample of domestic wood shavings and sawdust, accumulated over time from the chopping of firewood from nearby eucalyptus forests; this sample was collected from a property located on the edge of National Reserve in Golden Square, Bendigo, Victoria.

Approximately 0.5g of sample was placed in a 25mL conical flask. This was added 10mL of sterile deionised water, and the flask was placed on an orbital shaker at room temperature for 30 minutes. Serial dilutions of the water dispersion were prepared as follows:

0.9mL of sterile water was added into four 1mL sterile tubes. A sample of water (0.1mL) from the 10mL flask was added to the first tube. The contents of the tube were mixed well, and 0.1mL added to the second tube, and the procedure was repeated down to the fourth tube.

Samples (0.1 mL) from each tube was streaked onto Luria-Bertani agar. The agar plates were sealed and placed in a incubator at 37°C overnight. Colonies of bacteria appeared on the plates, and individual colonies were picked off and replated onto fresh Luria-Bertani plates.

The composition of Luria-Bertani medium is:

tryptone 10g
yeast extract 5g
sodium chloride 10g
deionised water 1L

For Luria-Bertani (LB) agar, 18g of agar is added to the above components. All media were sterilised by autoclaving at 121°C for 20 minutes.

The organism was isolated in pure culture, and a sample was deposited under the Budapest Treaty in the Australian Government Analytical Laboratories as described above.

The bacterium has the following taxonomic characteristics:

rod-shaped bacterium with a centrally-located spore
Gram positive
obligately aerobic

Example 2 Growth Conditions

The bacterium is not fastidious, and can be grown on a range of media, including LB broth. The requirements are:

1. a source of carbon, most conveniently a carbohydrate such as dextrose,
2. a source of nitrogen, most conveniently as a tryptone, and
3. complex nutrients, most conveniently as yeast extract.

The bacterium can be grown within the temperature range 20 to 45°C and within the pH range 5 to 9.5.

The bacterium can be grown successfully under different fermentation conditions, including solid state or submerged culture; fermentation continues under aerobic conditions with or without agitation.

Example 3 Production and Characterisation of Xylanase

When grown under the conditions described in Example 2, bacterium B698 synthesizes xylanase, and releases the enzyme into the extracellular medium. While xylanase is produced constitutively, addition of xylan to the culture medium as an additional carbon source further enhances the level of xylanase production. The added xylan may be in the form of isolated wood xylan, or may be a component of lignocellulosic material such as wheat bran.

Xylanase was assayed using the following conditions:

Substrate: 1% birchwood xylan
Buffer: 50mM sodium phosphate/citric acid, pH 6.
Incubation temperature: 50°C
Incubation time: 20 minutes

The enzyme reaction was stopped with 3,5-dinitrosalicylic acid (DNS) reagent which measures, using xylose standards, the amount of reducing sugar produced in 20 minutes. Enzyme units are expressed in nanokatals (nkats), where 1 nkat is the amount of xylanase which will produce 1 nmole of xylose per second under the defined conditions.

Example 4 Production of Xylanase by Submerged Fermentation

Xylanase from B698 can conveniently be prepared by submerged fermentation. B698 seed culture can be prepared overnight in LB broth at 37°C. This inoculum is added to an LB broth containing beechwood xylan (2% w/v). The pH of the broth is increased to pH 7.8 by the addition of 2M sodium hydroxide, and the temperature adjusted to 37°C. The broth is stirred (600rpm) and aerated with filtered sterile air (0.7 L of air/L of broth/min).

The seed inoculum is added to the broth and the above conditions of temperature, pH, agitation and aeration maintained. Samples of culture are taken at regular intervals to monitor the production of xylanase. Optimal levels of xylanase (7,300 nkat/mL) are obtained within 90 hours of fermentation.

Example 5 Characterisation of Xylanase

The crude enzyme preparation from the fermenter broth was characterised with respect to pH and temperature.

a) pH Optimum.

The xylanase activity was determined as described above, with the exception that the buffer was changed to obtain a stable pH. The results are listed in Table 1 below. The data is further expressed in Figure 1. The optimal pH for xylanase activity was found to be pH 6-6.5.

Table 1

pH Profile of B698 Xylanase

pH	Relative Xylanase Activity (%)
4	15
5	83
6	100
6.5	99
7	77
8	69
9	37
10	11

b) Temperature Optimum

The xylanase activity of B698 was determined as described above, except that the temperature was altered within the range from 40 to 80°C. Results are listed in Table 2 and further expressed in Figure 2. The optimal temperature for xylanase activity was found to be 60°C.

Table 2

Temperature Profile of B698 Xylanase

Temperature (°C)	Relative Xylanase Activity (%)
40	65
50	85
60	100
70	51
80	27

Example 6 Thermal Stability

The stability of B698 xylanase was determined at pH 6 and 60°C, the optimal pH and temperature respectively for the enzyme system. Samples were tested for residual activity at regular intervals as described in the xylanase

assay conditions above. After 3 hours at 60°C, 100% xylanase activity was retained. Even after 22 hours, 90% of the xylanase activity was retained. Thus, xylanase from B698 is very thermally stable.

Example 7 Stability at 4°C

The stability of B698 xylanase was determined at 4°C by storing it at that temperature. Samples were tested for activity at regular intervals under the conditions described in the xylanase assay conditions above. After 22 days, 100% of the original activity was retained.

Example 8 Variation During Fermentation

The characterisation of the xylanase system, as described above, was carried out during the first 40 hours of fermentation. When the fermentation proceeds beyond 40 hours, up to 110 hours, we have found that the xylanase system changes. For example, the xylanase activity at pH 5 drops to 60% of that at pH 6 (cf. 80% activity when measured within 40 hours). These results indicate that at least two different xylanases are produced during fermentation of B698.

Example 9 Use Of B698 Xylanase As A Bleaching Aid

The crude xylanase system (167nkat/g of pulp) was mixed with unbleached kraft pulp (35 g oven dried basis) at consistency 8% and adjusted to pH 6.3 with sulphuric acid. The mixture was incubated for 2 hrs at 50°C. The pulp was extracted with sodium hydroxide and bleached with either chlorine dioxide-sodium hydroxide-chlorine dioxide or hydrogen peroxide sequences. Results are listed in Tables 3 and 4.

Table 3

Bleaching of Kraft Pulp (DED bleaching)

	Bleaching Pulp		
Treatment	Brightness (% ISO)	Freeness (ml CSF)	Yield (%)
EDED	74.7	560	97.2
XEDED	77.3	560	97.5

Table 4

Bleaching of Kraft Pulp (P bleaching)

	Bleached Pulp		
Treatment	Brightness (% ISO)	Freeness (ml CSF)	Yield (%)
EP	53.6	505	98.4
XEP	56.1	510	97.4

D - chlorine dioxide, 2.5% as chlorine, 70°C, 2hr
E - sodium hydroxide, 1%, 50°C, 1hr
P - hydrogen peroxide, 1%, 1.5% NaOH, 3hr
X - Xylanase treatment

It is evident from these results that bleaching by either method in the presence of xylanase results in a yield comparable to that of the control, and improved characteristics of brightness and freeness compared to the control.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

CLAIMS

1. A bacterium, isolatable from wood compost, having the following characteristics:

A. Ability to grow at a temperature between 20 and 45°;

B. Ability to grow in the pH range of 5 to 9.5;

C. Ability to grow on Luria-Bertani agar at 37°;

D. Ability to grow under solid state or submerged culture conditions; and

E. Constitutive production and/or extracellular release of at least one xylanase having an associated cellulase activity of less than 0.1%.

2. A bacterium according to Claim 1, which is a Gram positive, obligately aerobic rod-shaped spore-forming bacterium, in which the spores are centrally-located.

3. A bacterium according to Claim 1 or Claim 2, in which production of the at least one xylanase is enhanced by growth in the presence of xylan or of a lignocellulose substrate.

4. A bacterium according to Claim 1, Claim 2 or Claim 3 in which the at least one xylanase has at least one characteristic selected from the group consisting of activity at pH between 4.5 and 9, a thermal activity range up to 80°C, and high thermal stability up to 70°C.

5. A bacterium according to any one of Claims 1 to 4, in which the at least one xylanase is effective on both soluble and insoluble xylans.

6. A bacterium having the characteristics of the bacterial isolate designated B698, as deposited in the Australian Government Analytical Laboratories under Accession No. N94/7647, or a mutant or derivative thereof having the ability to produce a xylanase as defined in any one of Claims 1 to 5.

7. A xylanase having associated cellulase activity of less than 0.1%, and which is produced by a bacterium as defined in any one of Claims 1 to 6.

8. A bleaching aid, bleach booster or paper deinking composition comprising a xylanase according to Claim 7, together with an industrially acceptable carrier.

9. A process for bleaching kraft pulp, comprising the step of using a xylanase according to Claim 7 as a bleaching aid or bleach booster.

10. A process according to Claim 9, carried out at neutral to alkaline pH and/or at a temperature of 40° to 80°C.

11. A process according to Claim 10, carried out at a temperature of 50° to 70°C.

12. A process according to Claim 11, carried out at a temperature of 50° to 65°C.

13. A process for removal of printing ink from paper, comprising the step of using a xylanase according to Claim 7.

14. A process for preparing an animal feed composition, comprising the step of adding a xylanase according to Claim 7 to said composition.

15. An animal feed composition comprising a xylanase according to Claim 7.

16. A method of preparing dough for bread making which comprises the step of incorporating a xylanase according to Claim 7 in said dough.

17. A dough for the preparation of bread, comprising a xylanase according to Claim 7.

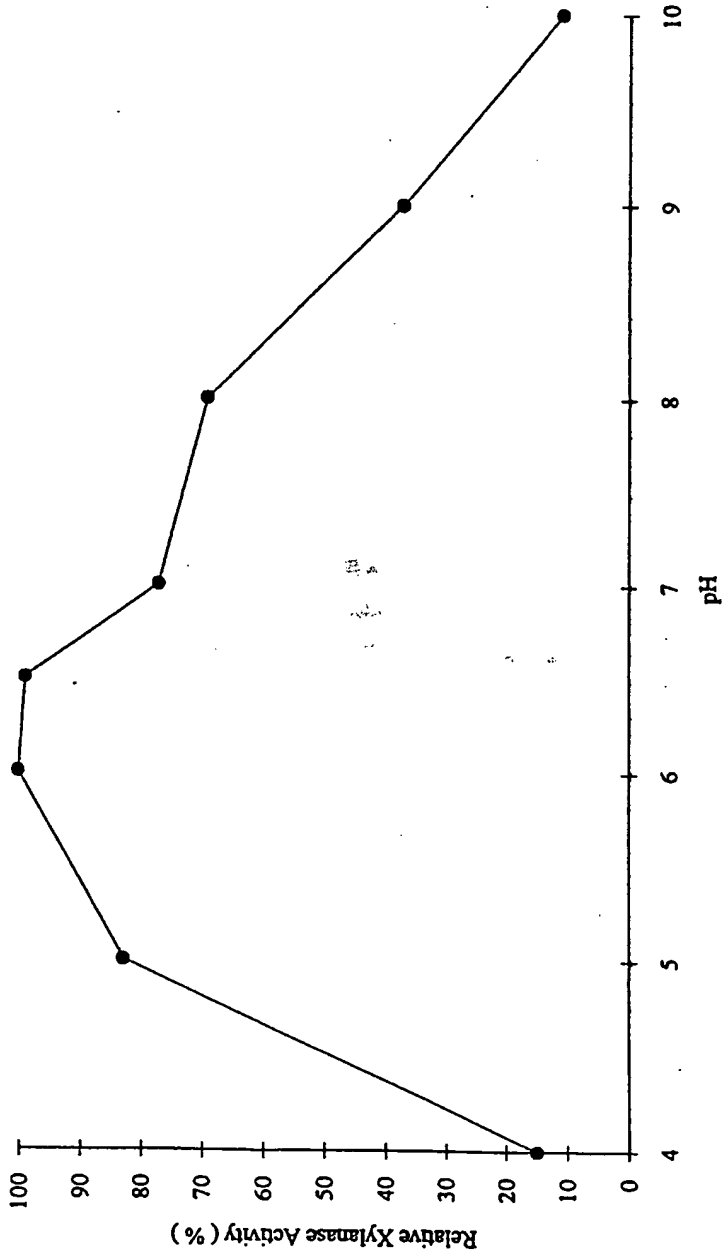
Relative Xylanase Activity (%)
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

FIGURE 1

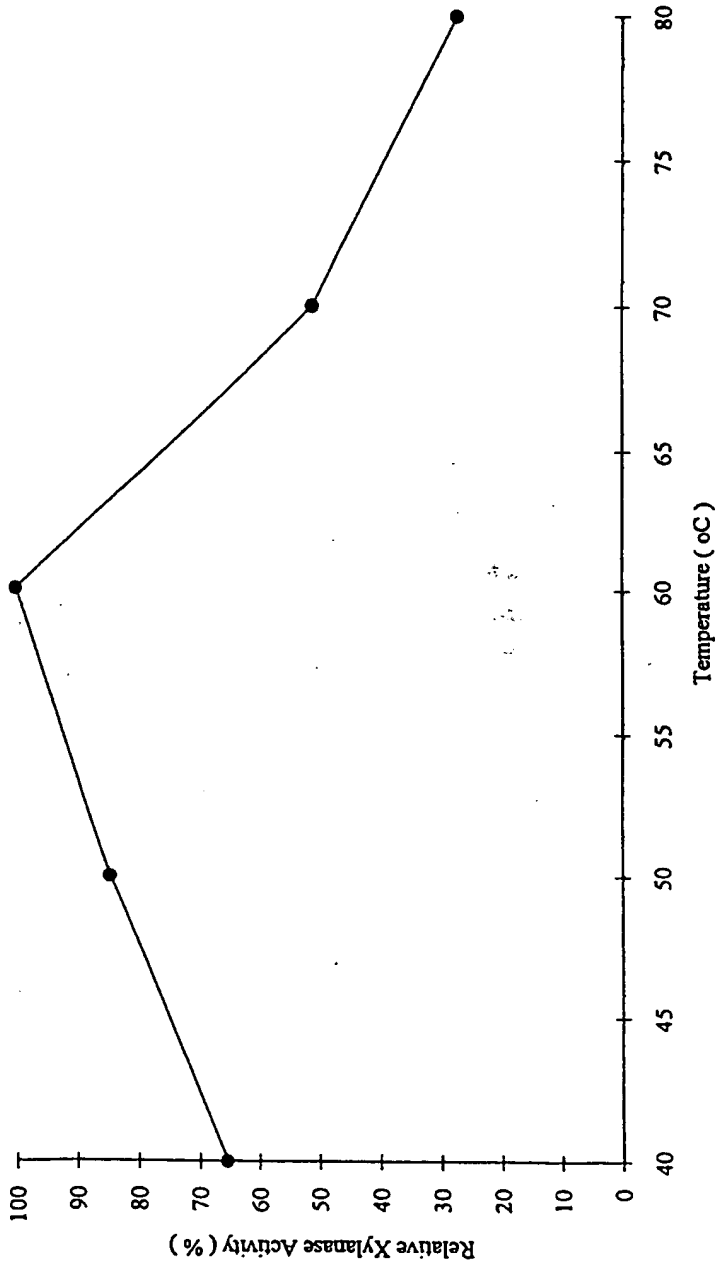
Relative Xylanase Activity (%)
100
90
80
70
60
50
40
30
20
10
0
Temperature (oC)
40
45
50
55
60
65
70
75
80

FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 95/00202

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[6] C12N 1/20; C12N 9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT AND CHEM ABS
SEE DETAILS IN ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPM, JAPIO
BIOTECH AND PAPERCHEM2 (SEE DETAILS IN ELECTRONIC DATABASE BOX BELOW)

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT WPAT, BIOTECHNOLOGY, CHEMICAL ABSTRACTS, JAPIO, USPM DATABASES; KEYWORDS: XYLANASE# AND BACTERI:
PAPERCHEM2 STN INTERNATIONAL DATABASE, KEYWORDS: AS ABOVE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	Enyme and Microbial Technology (April 1993), Vol. 15, Pages: 343-347 (Abhay Shendye and Mala Rao "Molecular cloning and expression of xylanases from an alkalophilic thermophilic Bacillus (NC1M59) in Bacillus Subtilis A8". See whole article.	1-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 1995	25 July 1995 (25.07.95)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer Arati Sardana ARATA SARDANA Telephone No. (06) 2832627

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	Canadian journal of microbiology (1993), Vol. 39, No. 12, pages 1162-1166 (A. Blanco and F.I.J PASTOR) "Characterization of cellulase - free xylanases from the newly isolated Bacillus sp. strain Bp-23". See whole article.	1-17
Y	The Journal of General Microbiology (1993), Vol. 139, pages 1987-1993 (P. WANG et al.) "Xylanases from Streptomyces cyaneus : their production, purification and characterization". See whole article.	1-17
Y	WO 94/04664 (NOVO NORDISK A/S) published 3 March 1994. See whole document.	1-17
P,A	US 5405769 (CAMPBELL et al.) published 11 April 1995. See whole article,	1-17

INTERNATIONAL SEARCH REPORT
Information on patent family member

International application No.
PCT/AU 95/00202

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	9404664	DK	1055/92	FI	950852
US	5405769	WO	9424270		

END OF ANNEX